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Clonal Forestry and Genetic Engineering: Forest Biotechnology — Where We Stand and Future Prospects and Impacts

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CLONAL FORESTRY AND GENETIC ENGINEERING: FOREST BIOTECHNOLOGY - WHERE WE STAND AND FUTURE PROSPECTS AND IMPACTS

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Summary Sentence: Impacts of clonal forestry and genetic engineering on the pulp and paper industries.

Executive Summary: Fiber supply needs can be met by clonal propagation and genetic transformation methods. For many commercially important angiosperm tree species these methods are relatively straightforward, and the first field tests of transgenic hardwoods are in progress. In contrast for the gymnosperms, especially loblolly pine, the techniques are limiting. Somatic embryogenesis is a propagation technique with great potential, but currently is limited by embryo quality; however, integrated molecular and cellular approaches, combined with tissue culture methods are being used to improve embryo quality. The tools for genetically engineering hardwoods and softwoods are being refined. The future impacts of clonal forestry and genetic engineering at the mill operations are discussed.

INTRODUCTION

The U.S. forest products industry is a major force in the U.S. economy, comprising approximately 7% of the U.S. manufacturing output and employing, mostly in rural communities, some 1.4 million people with a $46 billion annual payroll (1). The value of sales was about $400 billion in 1994 and by the year 2010, the world demand for paper is expected to increase nearly 50%, causing regional softwood and native hardwood supply constraints (2).

Currently, much of the U.S. industry faces an uncertain future with reduced availability of the timber resources that supply the industry's basic raw material needs. This potential shortage results from a continuous reduction in the forest landbase from which trees can be harvested due to environmental restrictions and urban growth and development. In addition, yield losses occur due to environmental stresses, pathogens, and pests. The slow continuous loss in forest acreage coupled with increasing pulp and paper demands and the availability of cheap fiber supplies from rapidly growing Brazilian and Southeast Asian tropical trees has put intense pressure on U.S. companies to increase the yield of wood/acre (3). If the industry is to continue to grow, it must sustain reliable low cost sources of raw materials.

The Forest Science and Technology Committee of the American Forest & Paper Association issued a report in late 1996, which says that the number one research priority of the U.S. forest products industry in the Far West, Intermountain, North Central, and Northeast regions of the United States is the sustainable growth and yield of trees (4). In the Southeast, where growth rates are the highest in the United States, the number one priority is sustainable site productivity. If productivity of U.S. forests cannot be increased within the next decade, it is likely that much of the industry will import raw materials or relocate production to foreign countries. This lends urgency to research efforts to protect forests from the damaging effects of pathogens and pests and environmental stress and to boost domestic forest productivity. These concerns with biomass availability and cost have lead the forest products industry to concentrate on improving tree growth.

Forestry-related research and development is focused on creating sustainable fiber farms or tree plantations. Farming trees with elite germplasms should increase growth rates and yields of wood/acre. Clonal propagation of high-value, fast-growing trees in a crop-like setting along with gene transfer to improve trees will decrease wood costs and increase wood quality. The combination of elite germplasm and clonal propagation offers the potential to sustainably meet future industry needs for high quality raw materials.

At IPST, the Forest Biotechnology Program is aligned to meet these needs by focusing on two major areas. Multiplication of high-value loblolly pine trees through somatic embryogenesis and genetic engineering of high value cottonwood trees.

Why focus on loblolly pine and cottonwood? Loblolly pine maintains a dominant position in the forest products industry over the Southeastern United States. Boyer and South found that in 1980 approximately 966 million loblolly pine seedlings were produced in the Southeastern U. S.; representing 60 percent of the tree seedlings produced in the U. S. that year (5). Southern pine plantations in 1990 composed approximately 34% of the 62 million acres classified as pine timberland (6). The latest Southern forest inventories in 1995 indicate approximately 199 million acres of active commercial timberland with 14% pine plantation, 18% natural pine forests, 15% in mixed oak and pine, and the remaining 53% as hardwood forest type (Personal communication, Raymond Sheffield). Thus, the percentage of intensively managed plantation pine increased from 34 to 44% from 1990 to 1995. This trend is expected to continue. Fast-growing cottonwood and poplar hybrids can produce some of the highest yields amongst U.S. hardwoods when grown in a temperate U.S. plantation setting (7). In addition, many species of cottonwood and poplars are easy to grow, can be propagated easily from rooted cuttings, and are amenable to tissue culture and genetic engineering techniques. IPST has chosen *Populus deltoides* to work with due to its potential value and application to Southeastern U.S. forests.

CLONAL FORESTRY AND SOMATIC EMBRYOGENESIS: WHERE DO WE STAND?

Planting forests with elite clonal germplasm will require efficient methods to multiply or copy high-value trees. In nature, plants come from seeds, which are shells containing a plant embryo and food materials to sustain the embryo during germination. During development, the embryo will grow and mature, eventually germinating out of the seed as a small plantlet that will root in the soil. The use of natural elite seed for mass plantings requires many years due to the long life cycle of trees. Consequently, methods for rapid vegetative propagation are preferred. Rooted cuttings have been used successfully to propagate some angiosperm and coniferous trees. However, it is currently difficult to produce large numbers of rooted cuttings of desirable loblolly pine trees. Presently, somatic embryogenesis is viewed as the most promising technology to multiply elite germplasm of loblolly pine in the future (8).

In the 1970's, it was observed that given the right culture conditions, embryos could be developed in the laboratory. The term somatic embryogenesis (somatikos; Greek, from the body) was coined for this process. Somatic embryogenesis offers the advantage of rapid embryo multiplication in a small space. Potentially, enough embryos could be produced in a 10 ft by 10 ft room to plant an area the size of Georgia. The disadvantage is that, while fairly efficient for many plants, the process is difficult in pine trees of commercial importance. Yields of embryos are low; their quality is poor; and there are problems in regenerating trees. Understanding and improving somatic embryogenesis is a major research effort at IPST. In addition to its practical application, somatic embryogenesis provides a regenerative tissue culture system needed for the genetic engineering of forest trees.

Plant tissue culture broadly refers to the techniques of growing plant tissues or parts on a nutrient medium containing minerals, sugars, vitamins, and plant hormones, all under sterile conditions. Somatic embryogenesis is a type of plant tissue culture that starts with a piece of donor plant and forms new embryos (9, 10).

Initiation. Conifer somatic embryogenesis currently involves the culture of zygotic seed embryos, usually from breeding programs, to start or initiate a culture. Many pine embryos undergo a natural multiplication process in the seed called cleavage polyembryony. In this process, several genetically identical embryos form in the immature seed. Usually one embryo outgrows the others and goes on to fill the seed. Through the use of plant hormones, we can continue cleavage polyembryony to form masses of early-stage somatic embryos (Figure 1).

Multiplication. After initiation, somatic embryos are moved to another medium to increase their numbers (11). This can be done on a gelled or liquid multiplication medium, which again contains plant hormones to continue cleavage polyembryony and somatic embryo multiplication. Liquid media have the advantages of increasing growth rates, decreasing variation, and being easy to automate for transfer from one medium to another. A 1-L flask, one-third full will often contain in excess of 10,000 early-stage somatic embryos. Cultures may replicate 2-6 times weekly, rapidly producing large numbers of genetically superior somatic embryos. The major advantage of this technology lies in its ability to multiply highly valuable genetic material, forming an unlimited number of identical somatic seedlings.

Maturation. Once a sufficient population of embryos has been grown on the multiplication medium, embryos are placed on a development and maturation medium (11). A different set of plant hormones and environmental cues is required to continue development of the early-stage somatic embryo. Figure 2 shows late-stage embryos of loblolly pine formed on a maturation and development medium.

Germination. The last step is embryo germination and acclimation to achieve growth out of the tissue culture vessel to produce somatic seedlings ready for planting in the field (Figures 3 and 4).

Cryogenic Storage. Cryogenic storage is used to maintain long-term cell viability at ultra low temperatures (Figure 5). Water is the major component of all living cells and must be present for chemical reactions to occur within a cell. During cryopreservation, water changes to ice and cellular metabolism ceases. Dehydration also occurs changing the concentration of salts and other metabolites, causing an osmotic imbalance that can be detrimental to cell recovery. These detrimental effects can be minimized by

controlling the rate of cooling, using cryoprotective agents, and maintaining appropriate storage temperatures and rates of re-warming. This technology allows cultures to be stored for long periods of time and retrieved when desired. During storage, active cultures can be used to produce embryos and seedlings for field testing. Years later, after field evaluation, desirable cultures can be retrieved and used for production of high-value clonal seedlings.

GENE EXPRESSION DURING PINE EMBRYOGENESIS

Improving the somatic embryogenesis process and embryo quality requires an understanding of embryogenesis in the natural state. Unfortunately, no sensitive method of monitoring the biochemical changes during the course of development has been available. Embryo development, be it for humans, or sheep or plants, is the result of a controlled program of gene activity. The products of these genes are usually proteins. These may be enzymes, which utilize resources for growth, or structural proteins, which go to form the cell walls or other constituents.

The cartoon in Figure 6 summarizes the principal events in going from gene activity to cell constituents. In the nucleus, we have the chromosomes composed of a wound up ribbon of DNA. DNA is a long chain of nucleotides, whose sequence forms a genetic code. Much of this sequence is random, but at various places, the subunits are arranged in a meaningful order; this is a gene. When talking of the genetic code, one often invokes the analogy of language, so we may think of the DNA as a string of random letters. If we traveled along a strand of DNA, we would find that at certain places these letters appear in a recognizable sequence to form a word or sentence. This is a gene. As we continue, the order breaks down until farther along, we encounter another word or sentence, a different gene. Thus, a gene is a physical entity; it is a piece of DNA whose constituent subunits are arranged in a meaningful order.

When a gene is active, the information is copied into a similar type of molecule, messenger RNA (mRNA). For each gene, there may be tens to thousands of mRNA copies made, depending on the gene. These messages are then translated by an enzyme complex called the ribosome. The ribosome reads the information in the message and uses it to construct functional proteins, which are the enzymes and structural proteins of the cell.

There may be about 50,000 different genes in a plant cell. Not all are switched on at the same time or in the same part of the plant. Different genes are switched on by different signals. Certain genes are switched on by light. We have seen this in high school biology projects. If a seed is germinated in the dark, the plant is white. If it is moved into the light, it quickly turns green. That is because light-responsive genes are activated (12). Similarly, defense and repair genes are turned on by drought, cold, or insect damage. Genes involved in embryogenesis respond to internal cues and are switched on at certain times during development to produce the enzymes and structural components needed. So if we grow a plant in the dark, it will contain different mRNA compared to a plant grown in the light. Similarly, if we take an embryo at an early stage of development and one at a late stage of development, there will be different mRNA, indicating different gene activity.

Development is the result of a controlled sequence of gene activity with genes turning on and off in a specific order. Understanding this process will allow us to modify our laboratory practices so that embryo development can be optimized. Differential display (Figure 7) can help us follow this process and provide a comprehensive picture of gene activity throughout development (13). In this method, we extract mRNA from early-and late-stage embryos (or alternately, from natural and laboratory embryos of the same stage). We use a primer to reverse transcribe a subset of the mRNA. Using a technique called polymerase chain reaction (PCR), which acts like a molecular photocopier, we can copy a specific message a million fold in just a few hours. By conducting this reaction in the presence of a radioactively labeled deoxyribonucleotide,

separating the products by gel electrophoresis and exposing the gel to X-ray film, these results can be easily viewed. The presence of a band on a gel shows that the mRNA is present; thus, the gene is active. No band indicates an inactive gene. Figure 7 shows one band, but this reaction occurs simultaneously for hundreds of molecules.

We can apply this technique to embryo development by isolating natural and laboratory embryos of the same developmental stage and performing differential display. Similarities and differences in which genes are on or off are observable (Figure 8). The bands on the gel are intact DNA molecules, and by aligning the X-ray film with the original gel, we can locate and cut out bands of interest. These may be cloned and their DNA sequence determined. By comparing these sequences with DNA sequences lodged in international computer banks, we can try to match the cDNAs with previously characterized genes. A match and tentative identification can help us infer the biochemical changes that are occurring within the embryo. For example, the presence of mRNA for a sugar hydrolyzing enzyme may indicate metabolism of that sugar at a certain point in development, and supplementing media with this sugar at that time might be productive. Differential display can be applied in a number of other ways; as a method of quality control, we can ensure that successful protocols are being practiced. Differential display may also be used in an objective way to analyze embryo development; stages can be identified based on a banding pattern rather than relying on an expert, subjective eye to assess their maturity. The greatest advantage of this technique is that it lets us 'see' and isolate copies of the spectrum of genes that are active in a developing embryo.

FOREST TREE GENETIC ENGINEERING

Future prospects for genetic engineering of forest trees are high. Genetic engineering already impacts our everyday lives in the food we eat, the clothes we wear, and the pharmaceutical chemicals we use. Through the use of genetic engineering techniques, individual genes from other plants or animals may be isolated from the donor organism and transferred to a target microbe, animal, or plant cell. Forest trees have

generally been more difficult to work with mainly due to their long generation times and life cycles. For example, compare the several month corn breeding cycle to the many year cycle of a loblolly pine tree.

Foreign genes can be transferred to a forest tree resulting in faster tree improvement and unique gene combinations unachievable by traditional tree breeding. The successful genetic engineering of a forest tree requires four factors. 1) A desirable gene must be identified and isolated from a donor organism. 2) A plant regeneration system from single cells or a small group of cells is needed. 3) A mechanism for inserting or transferring foreign DNA into a target cell is required. 4) Additional DNA sequences for regulating the target gene, e.g., a promotor, is necessary to cause the target gene to function in the proper tissue when and where desired.

Genes of interest can be divided into two categories. First, we have genes that govern agronomic traits or the ability of trees to grow rapidly. These genes are expected to lower the cost of wood. This category includes genes for disease and pest resistance or tolerance of environmental stresses such as drought or flooding. Genes that help a crop to grow more efficiently, such as herbicide tolerance, also fall into this group. Most forest tree transformation efforts have focused on improving agronomic traits, including herbicide tolerance for stand establishment, insect resistance with *Bacillus thuringensis* toxins and protease inhibitors, bacterial soft rot resistance, and male sterility to inhibit transfer of foreign genes into the natural tree population (14).

A second category includes genes for value-added traits that improve production efficiency, product quality, or product value, and are expected to increase the value of wood. Genes for reduced lignin content or lignin type which are more easily removed during pulping, fall into this category. Genes responsible for improved fiber characteristics would also be included here. Several value-added traits are already being actively researched in order to isolate valuable genes. Lower lignin content and altering lignin linkages have been

achieved through lowering the activity of cinnamyl alcohol dehydrogenease, an enzyme in the lignin formation pathway, resulting in transgenic model plants of tobacco and aspen (15, 16, 17).

We are carrying out fundamental research into the molecular mechanisms that regulate xylem cell differentiation and secondary cell wall properties. We know little about many of the basic mechanisms that control xylem cell differentiation, earlywood/latewood transitions, juvenile vs. mature wood properties, wood extractives, cellulose synthesis, microfibril angle, fiber cell length, cell wall thickness, as well as cell wall composition and organization. This multidisciplinary research program is being carried out with close collaboration between IPST biologists, chemists, engineers, and physicists. As basic regulatory mechanisms are defined, rational strategies for altering fiber properties will become more tractable.

IPST is well positioned for gene transfer work in loblolly pine with a somatic embryogenesis system which can regenerate plants from a small group of cells. Embryos can be repeatedly grown and converted to plants in greenhouse and field environments. IPST maintains a library of cryogenically stored conifer cultures capable of producing plants through somatic embryogenesis and is currently one of three laboratories worldwide that has reported the ability to repeatedly produce somatic seedlings of loblolly pine.

Several gene transfer systems are available for movement of foreign DNA into target plants. The most common method uses *Agrobacterium tumefaciens*. This soil-borne bacterium is able to naturally genetically engineer plants to create an environment in which the bacterium can thrive. Molecular biologists have altered this organism to insert target genes into plants, including conifers, without causing plant disease (18). IPST is currently working jointly with the N.C. State Forest Biotechnology Group to efficiently transfer foreign genes into conifers (19). Other methods for gene transfer in forest trees include particle bombardment, electroporation, and polyethylene glycol (20).

As described above, there are several well-established techniques for transferring DNA into plant cells. The efficient production of a protein encoded by this DNA, however, requires that the appropriate gene control elements be present. These elements will ensure that mRNA is produced under the correct circumstance (Figure 9) and that the mRNA is stable and translated properly. The promoter is a region of a gene that directs the synthesis of RNA. Usually positioned adjacent to the point where RNA synthesis begins, the promoter contains regions, which are recognized, specifically, by protein transcription factors present in certain cells. Consequently, although the same genes are present throughout the plant, a gene will only be transcribed in cells containing those transcription factors. Some transcription factors are ubiquitous, others are restricted to a tissue, or produced only at a certain time of development or in response to an environmental cue. Many experiments in plant gene transfer have been conducted using a promoter from a plant virus. This virus promoter was useful in that it functioned in most plant cells, but unforeseen variations in expression have now rendered it too unpredictable to be relied upon (21). In seeking alternative promoters for use in genetic engineering, we are investigating tree promoters that give a higher level of expression than the virus promoter, and we are determining elements that will ensure expression under stress conditions as well as those that target gene expression to particular tissues. Figure 9 indicates that additional factors may influence the production of proteins. We are currently investigating the effect of certain sequences on the stability of mRNA and the efficiency with which mRNA is translated under different conditions.

GENETIC ENGINEERING OF ANGIOSPERM TREES

The *Populus* species are the most intensively studied angiosperm forest trees. Tissue culture and genetic transformation methods for *Populus* species and hybrids, including *P. deltoides* (22, 23), *P. tremuloides* (24), *P. trichocarpa X deltoides* (25), and *alba X tremula* (23) have been published. The protocol developed here at the Institute of Paper Science and Technology for *Populus deltoides* is similar to many of those available (22). Figure 10 shows a transgenic cottonwood leaf expressing the β-d-glucuronidase gene under the control

of the CaMV 35S promoter. In the southern United States, Sweetgum is an important hardwood, and transgenic plants that are herbicide-tolerant are being field tested. Table 1 provides a list of trees that have been successfully genetically engineered for a desirable trait and have progressed to a field evaluation stage. Currently, the U.S. Department of Agriculture APHIS program regulates field testing of genetically engineered organisms and requires field permits before most organisms are released into the environment.

HOW WILL FOREST BIOTECHNOLOGY AFFECT MILL OPERATIONS?

As clonal propagation practices such as somatic embryogenesis and rooted cuttings come into full commercialization, the accomplishments of breeding and genetic engineering programs will have more rapid and larger impacts on the mill. Wood growth rates will increase as agronomic traits such as pathogenic fungi and bacteria, insect pests, and weeds come under control. With changes in adaptation to environmental stresses, more desirable commercial wood species will be able to grow on a wider array of soil types and planting sites. Overall wood procurement costs will decrease as wood grows faster on a smaller landbase and comes closer to the mill decreasing transportation costs. In addition, mixtures of high-value tree lines will be selected for desirable growth rates in specific growing regions and for wood quality characteristics that can be matched to product needs. One of the most promising but least understood benefits of clonal propagation will be due to wood uniformity. Day-to-day wood uniformity should help reduce variability at every step in the papermaking process. This together with controlled fiber properties that are engineered so that specific product needs are taken into account should lead to new, improved, and higher value wood and paper products in the future.

REFERENCES

1. American Forest and Paper Association (1993).
2. McNutt, J. A. and Rennel, J. *Pulp & Paper International.* 39:(2) 48 (1997).
3. Arnold, R. B. Pulp and Paper. *Pulp & Paper,* 69:135 (1995).
4. American Forest & Paper Association, Forestry Research Advisory Council. Report to the Secretary of Agriculture, (1996).
5. Boyer, J. N. and South, D. B. *Southern J. Applied For.*, 8: 67 (1984).
6. Brown, M. J. and McWilliams, W. H. Proceedings Southern Plantation Wood Quality Workshop: A Workshop on Management, Utilization, and Economics of the South's Changing Pine Resource, Athens, GA, USDA Forest Serv. Gen. Tech. Rep. SE-63, Ashville, NC, (1989).
7. Luke III, D. L. *TAPPI 1994 Biological Sciences Symposium Proceedings*, TAPPI PRESS, Atlanta, p. 5.
8. Becwar, M. R. and Pullman, G. S. In: Somatic Embryogenesis in Woody Plants. Vol. 3-Gymnosperms. Eds. S. Mohan Jain, P. K. Gupta, and R. J. Newton, Kluwer, The Netherlands, p. 287 (1995).
9. Tautorus, T. E., Fowke, L. C., and Dunstan, D. I., *Can. J. Bot.,* 69: 1873 (1991).
10. Gupta, P. K., Pullman, G., Timmis, R., Kreitinger, M., Carlson, W. C., Grob, J., and Welty, E. *Bio/technology*, 11: 454 (1993).
11. Pullman, G. S. and Webb, D. T. *TAPPI 1994 Biological Sciences Symposium Proceedings*, TAPPI PRESS, Atlanta, p. 31.
12. Terzaghin, W.B and Cashmore A.R. *Ann. Rev. Plant Physiol. Plant Mol. Biol.*,46: 445-474 (1995).
13. Liang, P. and Pardee, A.B. *Science*, 257:967, (1992).
14. Charest, P. J. *The Forestry Chronicle*. 72: 37 (1996).
15. Halpin, C., Knight, N. E., Foxon, G. A., Campbell, M. M., Boudet, A. M., Boon. J. J. Chabbert, B., Tollier, M-T, and Schuch, W., *Plant J.*, 6: 339 (1994).
16. Boudet, A. M. and Grima-Pettenati J. *Molecular Breeding*, 2:25 (1996).

17. Baucher, M., Chabbert, B., Pilate, G., Doorsselaere, J. van., Tollier, M. T., Petit-Conil, M., Cornu, D., Monties, B., Montagu, M. van, and Inze, D., *Plant Physiol.*, (1996).
18. Sederoff, R. R. In: Transformation of Plants and Soil Microorganisms. K. Wang, A. Herrera-Estrella, M. Van Montagu Eds., Cambridge University Press, Cambridge, p. 150 (1995).
19. Wenk, A. R., Whetten, R., Pullman, G. S. and Sederoff, R.R., *In Vitro,* 33: 55 (1997).
20. Van Doorsselaere, J., Van Der Jijnsbrugge, K., Baucher, J., Leple, J. C., Rhode, A., Van Montagu, M., and Inze, D. Agro. Food Ind Hi Tech, Nov/Dec: 15 (1993).
21. Benfey, P. N. and Chua, N. H., *Science,*250: 959 (1990).
22. Dinus, R., Stephens, C. J. and Chang, S., In Proceedings Southern Forest Tree Improvement Conference, p 210, (1995).
23. Block, M. D., *Plant Physiol.*, 93:1110 (1990).
24. Tsai, C., Podila, G. and Chiang, V. *Plant Cell Reports,* 14(2/3): 94 (1994).
25. Strauss, S. A., Knowe, S. A., and Jenkins, J., *Journal of Forestry*, 95: 12 (1997).

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FIGURE LEGENDS

Figure 1. An ovule of loblolly pine initiating a culture of somatic embryos

Figure 2. Somatic embryos of loblolly pine formed on maturation and development medium.

Figure 3. Converted somatic seedlings of loblolly pine growing in non-sterile soil in leach tubes.

Figure 4. Greenhouse grown somatic seedlings of loblolly pine.

Figure 5. From left to right. Cryostorage vat capable of storing 10,000 tubes, each containing 1 ml of embryogenic culture; liquid nitrogen supply tank; freezing chamber; and computer over printer and freeze rate controller.

Figure 6. Diagram showing the flow of genetic information in a plant cell. Genetic information in the DNA is copied into similar types of molecules called messenger RNA (mRNA). The instructions encoded in these molecules are used to synthesize a protein by polymerizing constituent amino acids. Because there are 20 commonly occurring amino acids, an enormous array of proteins can be made.

Figure 7. Messenger RNA molecules are shown as gray bars with different colored 'tails' to indicate sequence differences. Small colored arrows are PCR primers.

Figure 8. A schematic diagram of the strategy of comparing gene expression in somatic (Laboratory) embryos and zygotic (Natural) embryos. The gray panel at the bottom of the diagram represents an X-ray film of a differential display gel; the black bars are bands corresponding to cDNA molecules amplified by PCR.

Figure 9. The uppermost bar (gray and blue) represents a gene. The dark blue region is copied into RNA; the gray region to the left represents a promoter. The mid blue bars are mRNA; the red bodies upon them are ribosomes; the sky-blue G-shapes represent proteins.

Figure 10. Cleared transgenic leaf of *Populus deltoides* cultivar C-175 showing the blue colored product in mesophyll tissue that express the β-d-glucuronidase gene.

Table 1. U.S. field release permits issued for forest trees, USDA-APHIS database (http://www.aphis.usda.gov//bbep/bp/)

TREE SPECIES	PHENOTYPES	STATE
Poplar	MG[1] selectable marker	IA
Poplar	IR[1] lepidopteran	WI
Poplar	HT[1] glyphosate, phosphinothricin PQ[1] male sterile	OR
Poplar	HT glyphosate	OR
Sweetgum	HT 2,4-D	GA
American Chestnut	OO[1] Chestnut blight resistant	CT
Poplar[2]	IR cottonwood leaf beetle, Phratora leaf beetle	OR
Poplar[2]	FR[1] disease resistant general	OR
Poplar[2]	HT glyphosate IR general resistant insect VR[1] bacterial soft rot resistant	OR, WA

1. MG, marker gene, HT, herbicide tolerance; IR, insect resistance; PQ, product quality; OO, other; FR, fungal resistance; VR, virus resistance;
2. Status pending

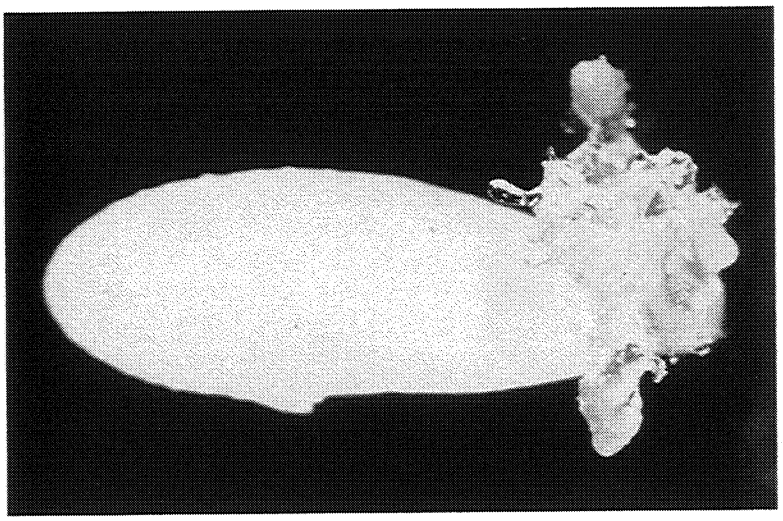

Fig. 1

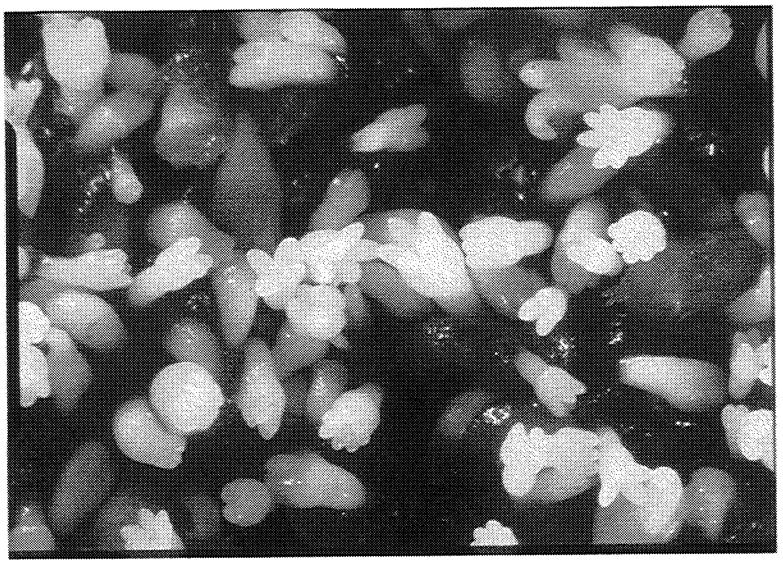

Fig. 2

Fig. 3

Fig. 4

Fig. 5

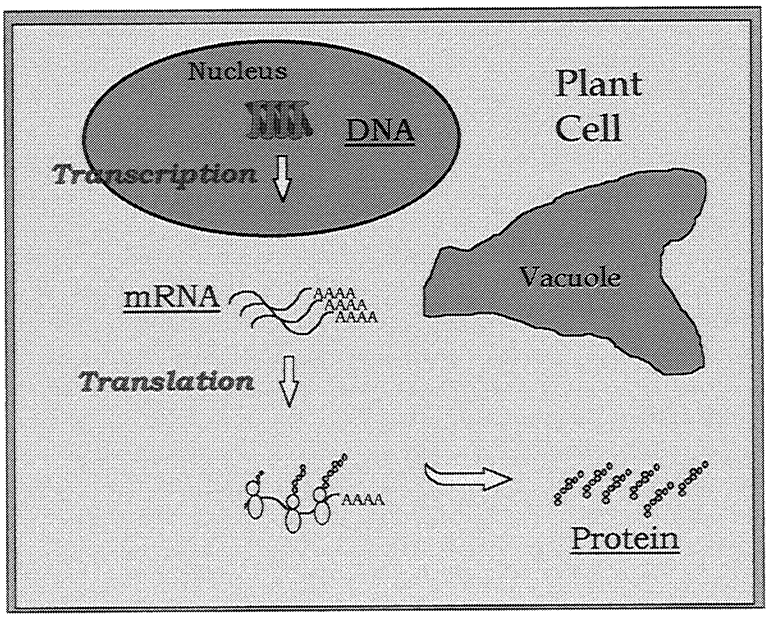

Fig. 6

Differential Display Principle

RNA is converted to cDNA by reverse transcription.

PCR multiplies a subset of the cDNAs.

The cDNAs are separated by electrophoresis and viewed.

When a band is present, the gene is "on", if absent it is "off".

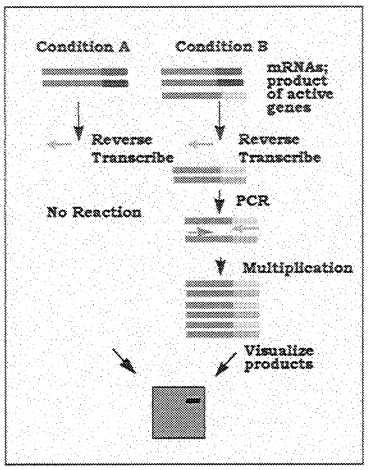

Fig. 7

Differential Display Can Be Used to Follow Gene Expression During Embryogenesis in Trees

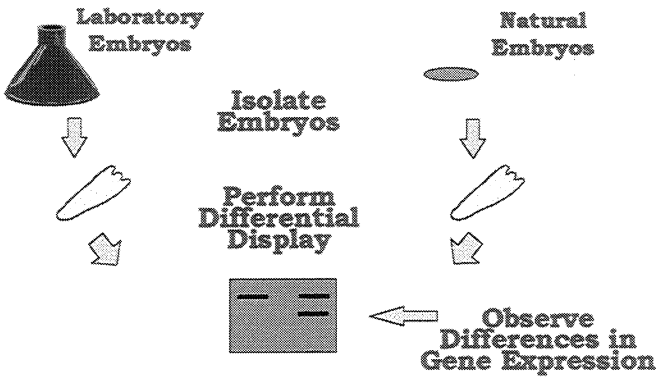

Fig. 8

Factors Affecting Gene Expression

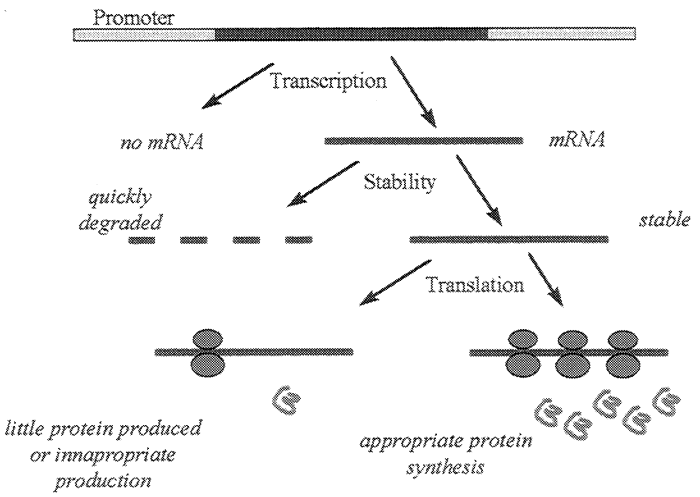

Fig. 9

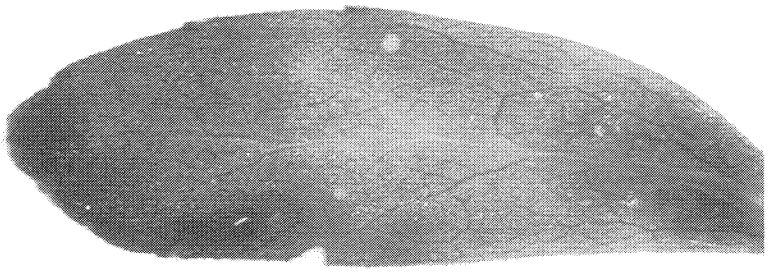

Fig. 10